

RESEARCH PAPER

Proteomic analysis of chromoplasts from six crop species reveals insights into chromoplast function and development

Yong-Qiang Wang^{1,*}, Yong Yang^{2,*}, Zhangjun Fei^{2,3}, Hui Yuan¹, Tara Fish², Theodore W. Thannhauser², Michael Mazourek¹, Leon V. Kochian², Xiaowu Wang⁴ and Li Li^{1,2,†}

¹ Department of Plant Breeding and Genetics, Cornell University, Ithaca, NY 14853, USA

² USDA-ARS, Robert W. Holley Center for Agriculture and Health, Cornell University, Ithaca, NY 14853, USA

³ Boyce Thompson Institute for Plant Research, Cornell University, Ithaca, NY 14853, USA

⁴ Institute of Vegetables and Flowers, Chinese Academy of Agricultural Sciences, Beijing 100081, China

* These two authors contributed equally to this work.

† To whom correspondence should be addressed. E-mail: ll37@cornell.edu

Received 6 November 2012; Revised 11 December 2012; Accepted 11 December 2012

Abstract

Chromoplasts are unique plastids that accumulate massive amounts of carotenoids. To gain a general and comparative characterization of chromoplast proteins, this study performed proteomic analysis of chromoplasts from six carotenoid-rich crops: watermelon, tomato, carrot, orange cauliflower, red papaya, and red bell pepper. Stromal and membrane proteins of chromoplasts were separated by 1D gel electrophoresis and analysed using nLC-MS/MS. A total of 953–2262 proteins from chromoplasts of different crop species were identified. Approximately 60% of the identified proteins were predicted to be plastid localized. Functional classification using MapMan bins revealed large numbers of proteins involved in protein metabolism, transport, amino acid metabolism, lipid metabolism, and redox in chromoplasts from all six species. Seventeen core carotenoid metabolic enzymes were identified. Phytoene synthase, phytoene desaturase, ζ -carotene desaturase, 9-*cis*-epoxycarotenoid dioxygenase, and carotenoid cleavage dioxygenase 1 were found in almost all crops, suggesting relative abundance of them among the carotenoid pathway enzymes. Chromoplasts from different crops contained abundant amounts of ATP synthase and adenine nucleotide translocator, which indicates an important role of ATP production and transport in chromoplast development. Distinctive abundant proteins were observed in chromoplast from different crops, including capsanthin/capsorubin synthase and fibrillins in pepper, superoxide dismutase in watermelon, carrot, and cauliflower, and glutathione-S-transferase in papaya. The comparative analysis of chromoplast proteins among six crop species offers new insights into the general metabolism and function of chromoplasts as well as the uniqueness of chromoplasts in specific crop species. This work provides reference datasets for future experimental study of chromoplast biogenesis, development, and regulation in plants.

Key words: Carrot, cauliflower, chromoplast, papaya, pepper, proteomics, tomato, watermelon.

Introduction

Flowering plants contain many types of plastids, such as chloroplasts in green tissues, chromoplasts in pigmented flowers, fruits, and roots, amyloplasts in storage tissues of seeds, tubers, and stems, and etioplasts in tissues grown in

dark. These plastids fulfil various distinctive and essential functions for plant growth and development. They serve as the main sites for photosynthesis and/or many important primary and secondary metabolism, including the synthesis of

starch, fatty acids, amino acids, and secondary metabolites. Plastid genomes of flowering plants encode approximately 100 proteins, and most plastid proteins are nuclear-encoded. The predicted proteome size for all plastid types ranges from 2000 to 3500 proteins (van Wijk and Baginsky, 2011). Studies on chloroplasts have long been the primary focus and chloroplast proteomic analysis has been extensively carried out (van Wijk and Baginsky, 2011). In contrast, relatively few proteomic studies on other types of plastids have been reported.

Chromoplasts are unique plastids that accumulate carotenoids, bringing vivid red, orange, and yellow colour to many flowers, fruits, and vegetables. Chromoplasts are frequently derived from fully developed chloroplasts as seen during fruit ripening from green to red fruits in tomato and pepper. In many cases, chromoplasts also arise from non-coloured plastids, such as those in carrot, sweet potato, and watermelon. Chromoplasts vary in the morphology of carotenoid-accumulating substructures and can be classified as globular, crystalline, membranous, fibrillar, and tubular (Egea *et al.*, 2010). Because chromoplasts accumulate carotenoids and other metabolites that are essential for nutritional and sensory quality of agricultural products, several proteomic studies on chromoplasts of fruits have been carried out, which identify 493 proteins from sweet orange (*Citrus sinensis*), 151 proteins from red bell pepper (*Capsicum annuum*), and 988 proteins from tomato (*Solanum lycopersicum*) (Siddique *et al.*, 2006; Barsan *et al.*, 2010; Zeng *et al.*, 2011). Proteomic analysis of the chloroplast to chromoplast transition in tomato identifies 1937 proteins (Barsan *et al.*, 2012). Analysis of chromoplast proteins from these fruits shows a range of metabolic processes in chromoplasts. The relative abundances of the identified chromoplast proteins differ considerably in comparison with the chloroplast proteome, showing chromoplast-specific metabolic processes (Siddique *et al.*, 2006; Barsan *et al.*, 2010). Furthermore, as chromoplasts from different fruits are derived either from chloroplasts or other non-coloured plastids and possess specific carotenoid-accumulating substructures, it is expected of unique features for chromoplast proteomes from different crop species.

Proteomics is a powerful approach for comprehensive characterization of plastids. To gain a general and comparative characterization of chromoplast proteomes, this study performed proteomic analysis of chromoplasts from six crop species: watermelon, tomato, carrot, orange curd cauliflower, red papaya, and red bell pepper (Fig. 1A). To facilitate the identification of relatively abundant proteins, chromoplasts were subfractionated into stromal and membrane fractions and separated by one-dimensional SDS-PAGE gel electrophoresis. Proteins in the visible bands were analysed using nano-liquid chromatography tandem mass spectrometry (nLC-MS/MS). A substantially large numbers of chromoplast proteins were confidently identified. Relative abundance of the identified plastid proteins from each crop species were determined based on protein abundance index emPAI value (Ishihama *et al.*, 2005). Comparative analysis of chromoplast proteomes among these six crops reveals some new insights into the general metabolism and

function of chromoplasts as well as the uniqueness of specific chromoplasts.

Materials and methods

Plant materials and carotenoid analysis

Fresh mature fruits of red watermelon (*Citrullus lanatus* subsp. *vulgaris*), red papaya (*Carica papaya*), red bell pepper (*Capsicum annuum*), and tomato (*Solanum lycopersicum*), as well as vegetables of carrot (*Daucus carota* subsp. *sativus*) and orange curd cauliflower (*Brassica oleracea* L. var. *botrytis*) (Fig. 1A) were either purchased from local supermarkets or grown in field at Cornell University according to standard production practice. Carotenoids from different fruits were extracted and analysed following the method as described (Li *et al.*, 2012)

Chromoplast isolation and subfractionation

Chromoplasts from these six crop species were isolated using sucrose gradient essentially as described by Barsan *et al.* (2010) and Tetlow *et al.* (2003) with minor modifications. Briefly, approximately 250 g fresh materials were ground in 600 ml ice-cold extraction buffer containing 50 mM HEPES (pH 7.5), 2 mM EDTA, 330 mM sorbitol, and 5 mM 2-mercaptoethanol. Suspensions were filtered subsequently with four and eight layers of cheese cloth and centrifuged at 5000 g for 5 min at 4 °C. The crude chromoplast pellets were gently suspended in 5 ml extraction buffer containing 50% sucrose (w/v), overlaid with a discontinuous sucrose gradient (50, 30, 17%, w/v, in extraction buffer), and centrifuged at 62,000 g for 45 min at 4 °C. Intact chromoplasts between layers of 50/30% and 30/17% were carefully collected, washed three times with over 10-fold dilution in extraction buffer, and centrifuged at 5000 g for 5 min to remove potential contaminations of cytosolic proteins and other organelles. The washed intact chromoplasts were stored at -70 °C until use.

To subfractionate chromoplasts, purified chromoplasts were suspended in 300 µl lysis buffer (25 mM tricine, 1 mM EDTA, pH 8.0) and ruptured by three cycles of freezing (in liquid N₂) and thawing (in 37 °C water bath). An aliquot (100 µl) was saved as total chromoplast samples. The rest was centrifuged at 200,000 g for 40 min at

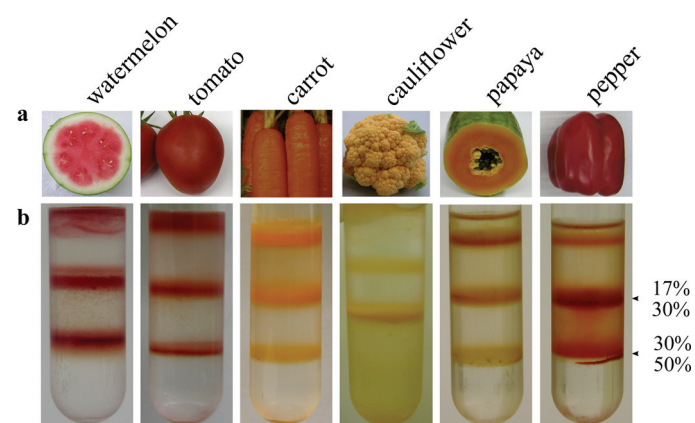


Fig. 1. Isolation of chromoplasts from six crop species. (A) Images of fruits and vegetables used for chromoplast isolation. (B) Chromoplasts were isolated by sucrose gradient centrifugation as described in Materials and Methods. Intact chromoplasts were collected from layers between 17/30% and 30/50% and washed extensively to remove contaminated proteins (this figure is available in colour at JXB online).

4 °C. Supernatant was carefully collected as stromal fraction. Pellet was washed twice in 1 ml lysis buffer to remove stromal proteins, and suspended in 100 µl lysis buffer as membrane fraction. All the operations were carried out at 4 °C or on ice.

Protein separation and nLC-MS/MS analysis by Synapt HDMS

Proteins of three chromoplast fractions (total, stroma, and membrane) from each species were separated on gradient SDS polyacrylamided gels (T = 9–15%, C = 2.6%) (18cm wide and 17cm high) and visualized by staining with Colloidal Coomassie blue (Invitrogen, Carlsbad, CA, USA). The visible gel bands from each lane were excised (Fig. 2). Subsequent in-gel digestion and peptide extraction were carried out as detailed previously (Yang *et al.*, 2007).

Tryptic peptide samples were analysed with a nanoACQUITY UPLC system coupled to a Synapt HDMS (Waters) equipped with a NanoLockSpray source as described previously (Yang *et al.*, 2011). Briefly, each reconstituted sample was injected onto a Symmetry C₁₈ trapping column and separated on a BEH C₁₈ RP column. The eluted peptides were detected by Synapt HDMS through a nano ion source (NewObjective, Woburn, MA, USA). The instrument was operated in data-dependent acquisition (DDA) mode. For each DDA cycle, five highest intensity ions exhibiting multiple charge states (2⁺, 3⁺ and 4⁺) were selected for MS/MS. The spectral acquisition time in each MS and MS/MS scan was 1s with a 0.02 s interscan delay. The mass range was set to m/z 300–1400 for each MS survey scans, and at m/z 50–1800 for MS/MS scans. All data were acquired by MassLynx v4.1 (Waters). Proteomics analysis was repeated with two different biological replicates.

Data analysis

All of the raw MS and MS/MS data were output as PKL files using the ProteinLynx Global Server 2.4 (PLGS, Waters). The subsequent database searches were carried out by Mascot Daemon (version 2.3, Matrix Science, Boston, MA) against specified database of each crop species of watermelon (Guo *et al.*, 2012), papaya (Ming *et al.*, 2008), tomato (The Tomato Genome Consortium, 2012), and cauliflower whole-genome CDS generated by the Institute of Vegetables and Flowers, Chinese Academy of Agricultural Sciences, China. The red bell pepper and carrot samples were searched against NCBI nr/ GreenPlants database (www.ncbi.nlm.nih.gov, downloaded on 28 February 2012). The default search settings used for the Mascot analysis were defined as described previously (Yang *et al.*, 2011). To reduce the probability of false identification, only peptides with significance scores at the 99% confidence interval were counted as identified.

Protein annotation, plastid localization prediction, and functional classification

All the proteins identified from each band were combined. Functional annotation of unique proteins was obtained based on information from local Blast (NCBI BLAST+, version 2.2.25 search against GenBank Green Plants database (<ftp://ftp.ncbi.nlm.nih.gov/genbank/>)). The functional description of individual protein was taken from the hit with lowest Expect value.

To predict the plastid localization of the identified proteins, a local Blast search was performed against *Arabidopsis* proteins (TAIR 10) and the proteins with lowest Expect value were chosen as *Arabidopsis* homologues. The corresponding AGI accession numbers were used to search against three databases. These databases included: gene models where their Gene Ontology (GO) terms were annotated as plastid localization in TAIR10 (www.arabidopsis.org); experimentally annotated plastid proteins from the PPDB database (<http://ppdb.tc.cornell.edu>); and proteins predicted to be plastid proteins by any program from SUBA database (<http://suba.plantenergy.uwa.edu.au>).

Protein functional classification and assignment were performed according to the bincodes of MapMan (<http://mapman.gabipd.org/web/guest/mapman>).

Results

Distinctive carotenoid profiles of various fruits and vegetables

Fruits of watermelon, tomato, papaya, red bell pepper, orange curd cauliflower, and carrot exhibit distinctive colours (Fig. 1A). HPLC analysis was carried out to determine the composition of carotenoids in the samples used in this study. As shown in Supplementary Fig. S1 (available at JXB online), watermelon and tomato accumulated predominantly lycopene in their fruits, which resulted in red colour. Carrot accumulated mainly β-carotene and α-carotene and cauliflower contained β-carotene. The accumulation of these carotenes was consistent with their orange colour of root or curd. Red-fleshed papaya accumulated several carotenoids with lycopene, β-carotene, and β-cryptoxanthin as the main components. Red bell pepper contained primarily capsanthin, which gave red fruit colour. Clearly, the distinctive colour of these fruits and vegetable owes their hues to the accumulation of those specific carotenoids.

Isolation of intact chromoplasts

A number of methods have been reported to isolate chromoplasts (Tetlow *et al.*, 2003; Barsan *et al.*, 2010; Zeng *et al.*, 2011). Crude chromoplasts from various crops were isolated following the methods of Barsan *et al.* (2010) and purified with a discontinuous sucrose gradient according to Tetlow *et al.* (2003). Intact chromoplasts that were accumulated as sharp bands in the interfaces of 17/30% and 30/50% sucrose were carefully collected (Fig. 1B). The broken plastids were either floating on top of the sucrose gradient or in the bottom of the centrifuge tubes. To remove potential contamination of cytosolic proteins and other organelles, the isolated intact chromoplasts were washed extensively in large volumes of extraction buffer. The chromoplasts purified via the approach used has been shown to be essentially free of cytosolic and mitochondrial marker enzymes (Tetlow *et al.*, 2003).

Overall proteomics analysis

The subfractionated chromoplast proteins from each crop species were separated on 1D SDS-PAGE gels (Fig. 2). Discrete and clearly visible bands from each chromoplast sample were cut out and analysed to provide a general survey of the chromoplast proteins and the relatively abundant proteins from different crop species. Up to 100 proteins with peptide significance scores at 99% confidence interval were identified from each gel band. Some proteins were detected in multiple gel bands as observed in other studies (Siddique *et al.*, 2006). Analysis of stromal and membrane fractions identified a total of 1170 distinct proteins for watermelon, 953 proteins for tomato, 1891 proteins for carrot, 2262 proteins for cauliflower, 1581 proteins for papaya, and 1752 proteins for pepper (Table 1 and Supplementary Table S1, available at JXB online).

By blasting against three databases (i.e. TAIR, PPDB, and SUBA), from 56.7% in papaya to 92% in pepper of the

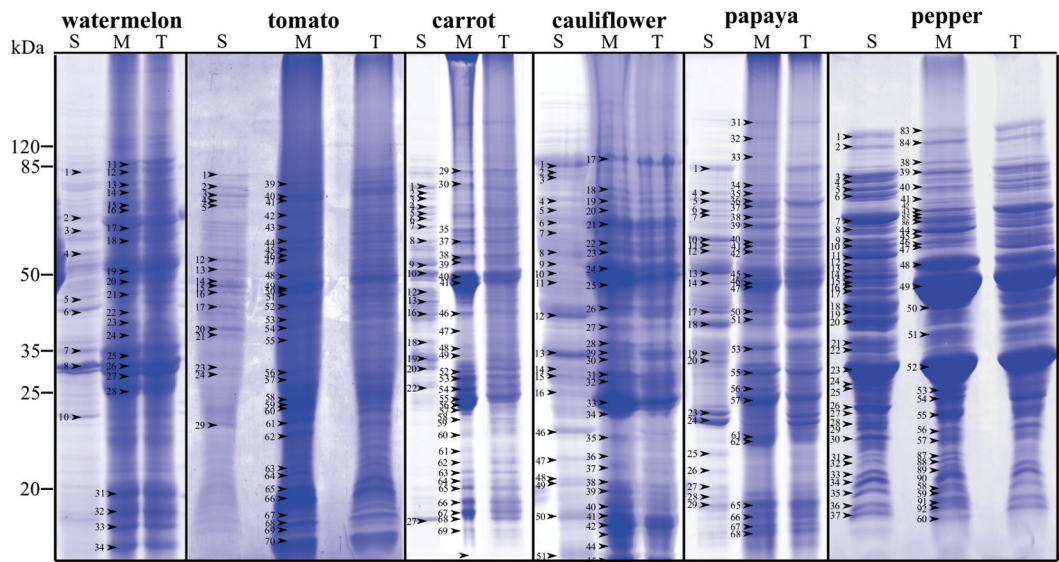


Fig. 2. One-dimensional SDS-PAGE gels of chromoplast proteins from various fruits and vegetables. M, membrane fraction; S, stromal fraction; T, total chromoplast (this figure is available in colour at *JXB* online).

identified proteins were predicted to be plastid localized by at least one prediction program (Table 1). Except in pepper, predicted plastid proteins from the other crop species contained an approximately 60% of total identified proteins, consistent with a recent study of tomato plastid samples (Barsan *et al.*, 2012). The final predicted plastid proteins ranged from 588 in tomato to 1612 in pepper (Table 1 and Supplementary Table S1). Analysis of proteins from stromal and membrane fraction revealed that a fraction of the proteins were localized in both fractions. The numbers of dual localized proteins varied from 7% in carrot to 18% in cauliflower (Table 1).

Cellular processes shared among chromoplasts from various crop species

The identified plastid proteins from different crops were categorized into functional groups using MapMan. The distribution of protein abundance in most functional classes was found to be similar among various fruits and vegetables

(Fig. 3). In general, apart from the group without an assigned function, the functional group associated with protein metabolism and process represented the most abundant group of proteins. Proteins associated with photosynthesis and carbon metabolism, electron transport/ATP synthesis, lipid metabolism, amino acid metabolism, stress and redox, signalling, and transport were also present in relatively high abundance.

Proteins involved in photosynthesis were detected in chromoplasts from all six crop species (Fig. 3 and Supplementary Table S1). A number of Calvin cycle proteins (i.e. chaperonin 60 α , phosphoglycerate kinase, fructose-bisphosphate aldolase, and transketolase) were consistently detected in chromoplasts from five to all six species (Supplementary Table S2). The persistence of Calvin cycle as well as oxidative pentose phosphate pathway proteins (i.e. transketolase) may provide intermediates for glycolysis and a main source of reducing power for various processes, respectively, in chromoplasts. Glycolysis also occurs in plastids in plants (Plaxton, 1996). Several glycolysis enzymes including aldolase, phosphoglycerate kinase,

Table 1. Summary of identified chromoplast proteins from six crops species.

	Watermelon	Tomato	Cauliflower	Carrot	Papaya	Pepper
Identified proteins						
Total	1170	953	2262	1891	1581	1752
Stroma	224	83	634	621	493	828
Membrane	797	783	1227	1137	777	650
Both	149	87	401	133	311	274
Predicted in plastid	719 (61.5)	588 (61.7)	1414 (62.5)	1252 (66.2)	896 (56.7)	1612 (92)
Identified proteins predicted in plastid						
Stroma	143	66	419	459	297	772
Membrane	476	466	734	699	430	577
Both	100	56	261	94	169	263

Values are *n* or *n* (%).

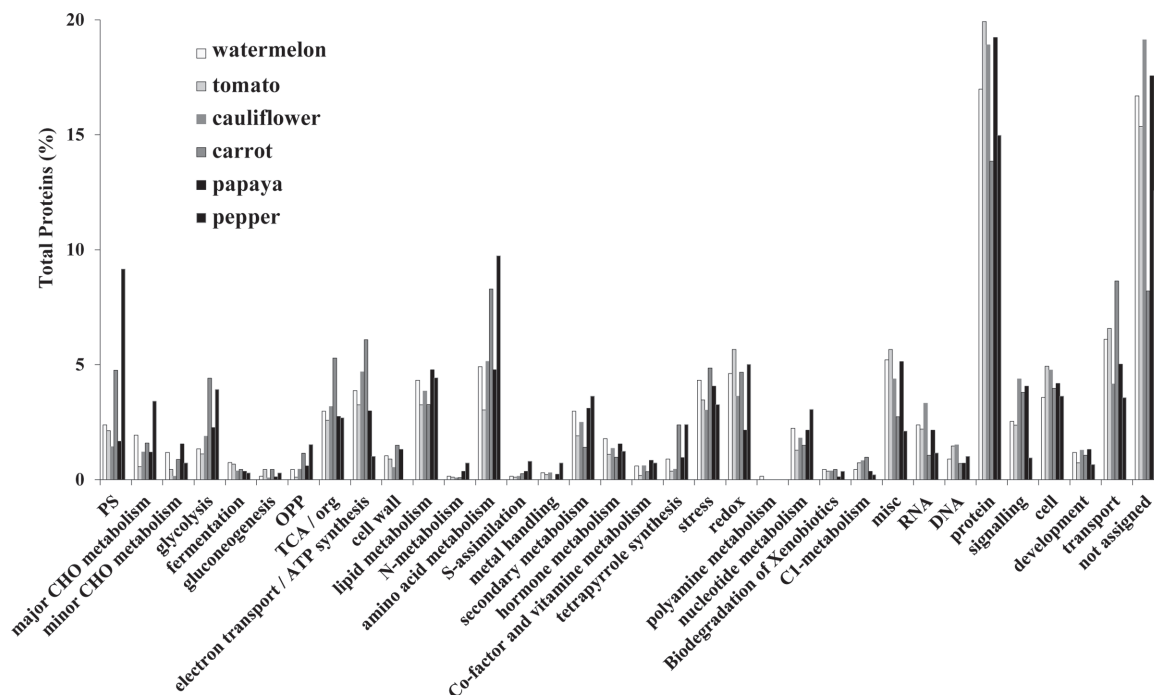


Fig. 3. Functional category of plastidial proteins identified from six crop species according to MapMan.

enolase, pyruvate kinase, and triosephosphate isomerase were observed in chromoplasts from five or all species. The observation of many glycolysis enzymes suggests an active metabolism in chromoplasts to generate energy, reducing power, and precursors for the synthesis of metabolites.

Fatty acids are synthesized in plastids. Noticeably, some enzymes for the synthesis of fatty acids as well as sulpholipids and glycolipids, such as NAD(P)-binding Rossmann-fold superfamily protein, thioesterase superfamily protein, sulphoglycerol, and FAD-dependent oxidoreductase were identified in chromoplasts from five or all crop species (Supplementary Table S2). The apparently universal existence of these proteins suggests the ability of the chromoplasts in synthesizing fatty acids and also forming various types of lipids, probably associated with the intense vesicular activity during chromoplast development as proposed by Barsan *et al.* (2010). Furthermore, key enzymes involved in lipid catabolism and essential for lipid homeostasis, such as AMP-dependent synthetase and ligase, long-chain fatty acid acyl-CoA synthetase, phospholipase D α 1, and multifunctional protein 2 were also repeatedly detected in chromoplasts from different crops. Dynamic activities of lipid metabolism appear to occur in chromoplasts.

Plastids are also the site for amino acid synthesis. Proteins involved in amino acid metabolism constituted one of the top three functional groups with most abundant numbers of proteins (Fig. 3). Many proteins in amino acid metabolism pathway were encountered, including oxidoreductase, methionine synthase, aspartate-semialdehyde dehydrogenase, ketol-acid reductoisomerase, isopropylmalate dehydrogenase 2, and 3-dehydroquinate synthase in chromoplasts from all six crop species (Supplementary Table S2). These results were

consistent with other reports (Siddique *et al.*, 2006; Barsan *et al.*, 2010; Zeng *et al.*, 2011). The persistent detection of these enzymes involved in the formation of an early branch point from aspartate and in the biosynthesis of branched-chain amino acids, aromatic amino acids, and methionine suggests their active synthesis within chromoplasts.

Redox systems are involved in the control of gene expression, protein import, enzyme activities, and repair mechanisms in plastids (Balsara *et al.*, 2010). Many key components in maintaining cell redox homeostasis were found in chromoplast proteomes from five or all six crop species (Supplementary Table S2). They include thylakoidal ascorbate peroxidase, monodehydroascorbate reductase, glutathione reductase, glutathione peroxidase, 2-cysteine peroxiredoxin B, and superoxide dismutase as shown in the chloroplast thylakoid proteome (Friso *et al.*, 2004). Further, a ubiquitous redox enzyme PDI-like that catalyses dithiol-disulphide exchange reactions as well as enzymes involved in ascorbate biosynthesis (L-galactono-1,4-lactone dehydrogenase) and glutathione biosynthesis (glutamate-cysteine ligase) were repeatedly identified. The persistent detection of a large number of proteins in the redox systems was consistent with previous studies (Barsan *et al.*, 2010; Zeng *et al.*, 2011), suggesting an important role of redox during chromoplast development.

Translocation of proteins into plastids is of importance for chromoplast biogenesis (Egea *et al.*, 2010). Consistent with detection of chaperone proteins which drive protein import into plastids, a number of translocons including Toc75 and Tic110 in the protein import machinery, were identified in chromoplast proteomes from nearly all crop species (Supplementary Table S2). Toc75 forms the main protein translocation channel of the Toc complex and is an abundant

protein in the outer envelope membrane (Eckart *et al.*, 2002). Tic110 plays a general role in protein import as a common component of the Tic complex (Inaba *et al.*, 2005). These two proteins likely constitute an important part of the protein import machinery in chloroplasts. Additionally, proteins involved in protein synthesis and degradation such as ribosomal proteins, GTP binding elongation factor Tu family protein, peptidase, aspartic proteinase, Clp proteases, and FtsH proteases were also persistently present. Proteins associated with protein translocation, synthesis, and degradation represent the most abundant functional group and constitute 14–20% of total plastid proteins, indicating active protein metabolism within chloroplasts.

Chloroplasts harbour a wide range of metabolic processes. Transportation of energy and various intermediates or substrates is essential for plastid development. A large number of transport proteins including V-ATPase, adenine nucleotide translocator, ABC transporter, glucose-6-phosphate/phosphate translocator, substrate carrier family protein, and S-adenosylmethionine carrier protein were persistently identified in chloroplast proteomes from nearly all crop species (Supplementary Table S2). The plastidial adenine nucleotide translocator catalyses the uptake of ATP, which affects energy-dependent metabolic processes and protein import in plastids (Neuhaus *et al.*, 1997). Glucose-6-phosphate/phosphate translocator mediates the import of carbon, which provides the substrate for the generation of reducing power and metabolite biosynthesis in non-green plastids (Flugge *et al.*, 2011). The detection of a range of transporters implies their importance in interconverting energy and transporting various substrates for energy and metabolite metabolism in chloroplasts.

Proteins involved in carotenoid biosynthesis and accumulation

There are over 20 core enzymes involved in carotenoid metabolism from the 2-C-methyl-D-erythritol-4-phosphate (MEP) pathway to carotenoid biosynthesis and degradation pathway (Fig. 4). Proteomic analysis of chloroplast proteins from various crop species identified 17 enzymes in the pathway. While ζ -carotene desaturase (ZDS) was detected in chloroplast proteomes from all crop species, a number of other enzymes, including 4-hydroxy-3-methylbut-2-en-1-yl diphosphate synthase (HDS), phytoene synthase (PSY), phytoene desaturase (PDS), 9-*cis*-epoxycarotenoid dioxygenase (NCED), and carotenoid cleavage dioxygenase 1 (CCD1), were identified in most crops (Fig. 4). In contrast, enzymes catalysing α - and β -carotene to xanthophyll biosynthesis were not detected in most of crops except pepper. Four core enzyme proteins (i.e. LCY-e, CYP97A, CYP97C, and NXS) were not identified in any crops (Fig. 4).

Carotenoids are sequestered in carotenoid-lipoprotein substructures, which are composed of carotenoids, lipids, and proteins (Li and Van Eck, 2007). Fibrillin is known to be the main component of carotenoid lipoprotein sequestration structures in pepper and contributes to high levels of carotenoid accumulation (Deruere *et al.*, 1994). Fibrillin and

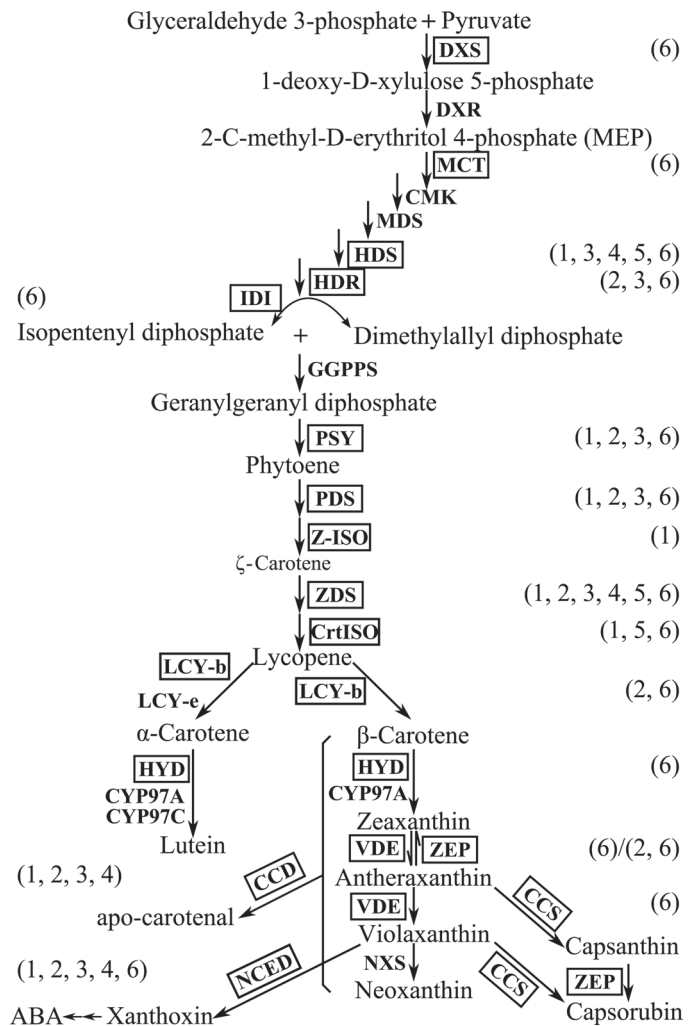


Fig. 4. Simplified scheme of carotenoid biosynthetic pathway. The enzymes identified in the chloroplast proteomes are boxed. The crop species representing by numbers that contain the particular carotenoid pathway proteins are aligned next to the enzymes in the pathway: 1, watermelon; 2, tomato; 3, carrot; 4, cauliflower; 5, papaya; 6, pepper. ABA, abscisic acid; CCD, carotenoid cleavage dioxygenase; CCS, capsanthin/capsorubin synthase; CMK, 4-(cytidine 5'-diphospho)-2-C-methyl-D-erythritol kinase; CrtISO, ζ -carotene isomerase/carotenoid isomerase; CYP97A, β -carotene hydroxylase (P450); CYP97C, ϵ -carotene hydroxylase (P450); DXR, 1-deoxy-D-xylulose-5-phosphate reductase; DXS, 1-deoxy-D-xylulose-5-phosphate synthase; GGPPS, geranylgeranyl diphosphate synthase; HDR, 4-hydroxy-3-methylbut-2-enyl diphosphate reductase; HDS, hydroxymethylbutenyl diphosphate synthase; HYD, β -carotene hydroxylase gene; IDI, isopentenyl diphosphate isomerase; LCY-b, lycopene β -cyclase; LCY-e, lycopene ϵ -cyclase; MCT, 2-C-methyl-D-erythritol 4-phosphate cytidyltransferase; MDS, 2-C-methyl-D-erythritol-2,4-cyclodiphosphate synthase; NCED, 9-*cis*-epoxycarotenoid dioxygenase; PDS, phytoene desaturase; PSY, phytoene synthase; VDE, violaxanthin de-epoxidase; ZDS, ζ -carotene desaturase; ZEP, zeaxanthin epoxidase; Z-ISO, ζ -carotene isomerase.

fibrillin homologues were also identified in the chromoplast of various crops (Table S1). Further, a number of plastid-lipid-associated proteins were also detected. They likely contribute to carotenoid accumulation in various types of chromoplasts.

Abundant proteins are detected in chromoplasts of various crops

Although chromoplasts from different crop species share extensively similar metabolic processes for chromoplast development and pigment accumulation, distinctive protein distribution patterns on SDS-PAGE gels were observed for chromoplast proteins (Fig. 2). A number of intensely staining gel bands in stromal and membrane fractions of chromoplasts from each crop species showed up. The protein abundance in each crop species and in each individual band was determined based on emPAI (Ishihama *et al.*, 2005; Yang *et al.*, 2007). Some of the abundant proteins identified from the major gel bands of each species are listed in Table 2, which includes both predicted plastid and non-plastid proteins.

In watermelon, the relatively most abundant proteins included formate dehydrogenase, Mn superoxide dismutase, Cpn20, ATP synthase, adenine nucleotide translocator, V-ATPase, and mitochondrial processing peptidase. In addition, a number of predicted non-plastidial proteins, such as annexins, were found at high abundance in watermelon chromoplast samples. The plastid formate dehydrogenase has been suggested to provide a ready source of high energy electrons to facilitate reduction reactions (Herman *et al.*, 2002). Mn superoxide dismutase functions in maintenance of redox homeostasis. Cpn20 is a small molecular chaperone and important in the maintenance of cellular homeostasis (Koumoto *et al.*, 1999). V-ATPase is found spanning the membranes of many organelles, which couples the energy of ATP hydrolysis to transport protons across membranes and acts an important regulator of membrane trafficking (Schumacher and Krebs, 2010). ATP synthase produces energy and transports protons. Adenine nucleotide translocator is also known as ADP, ATP translocator and mediates ADP and ATP exchange, including the import of ATP in non-green plastids (Flugge *et al.*, 2011). The high abundance of these proteins involved in energy metabolism and transport as well as in maintaining redox homeostasis implies their roles in watermelon chromoplast development.

In tomato, some of the most abundant proteins included V-ATPase, ATP synthase, adenine nucleotide translocator, ADP-ribosylation factor, small heat shock protein, alcohol dehydrogenases, and lipoxygenase homology domain-containing protein 1 (Table 2). These protein localization predictions were consistent with those from a recent study of plastid development in tomato (Barsan *et al.*, 2012). A number of predicted non-plastidial proteins including histone H4 were found to be present at high abundance in tomato chromoplast samples. Like the case for watermelon, the proteins involved in energy production and transport (i.e. ATP synthase and ADP/ATP translocator) as well as those involved in the production of reducing power (i.e. alcohol dehydrogenases) were among the most abundant proteins. Vesicle fusion is

important for chromoplast membrane formation (Huguene *et al.*, 1995). ADP-ribosylation factor, a small GTP-binding protein functioning as regulator of vesicular traffic (Memon, 2004), was identified to be abundant, indicating potentially active membrane proliferation in tomato chromoplasts. Lipoxygenases play an important role in the generation of fatty acid-derived flavour compounds (Chen *et al.*, 2004). The presence of high abundance of lipoxygenases may contribute to the production of a large number of flavour compounds during tomato fruit ripening.

Carrot is one of the richest sources for carotenes and develops chromoplasts underground. Superoxide dismutase, ATP synthase, V-ATPase, adenine nucleotide translocator, and NADH dehydrogenase, along with non-plastidial calmodulins, were highly abundant in carrot chromoplast samples (Table 2). ATP synthase and adenine nucleotide translocator were present in highest abundance in the membrane fraction of carrot chromoplasts. The prevalence of these proteins indicates the high requirement of energy for carrot chromoplast development. The presence of abundant plastidic Cu-Zn superoxide dismutase (Huang *et al.*, 2012) suggests the importance of maintenance of redox homeostasis in carrot chromoplasts.

Orange curd cauliflower results from a single gene (*Or*) mutation that changes the plastid type from leucoplasts to chromoplasts (Lu *et al.*, 2006). Several proteins such as malate dehydrogenase, Mn superoxide dismutase, V-ATPase, ATP synthase, adenine nucleotide translocator, mitochondrial processing peptidase, and 60S acidic ribosomal protein P2-2 were found to be abundant in orange cauliflower chromoplast samples (Table 2). As in carrot, ATP synthase and ADP/ATP translocator were among the most abundant proteins. Proteins involved in maintaining the optimal ratio between ATP and reducing equivalents (i.e. malate dehydrogenase) (Berkemeyer *et al.*, 1998), were also present at high abundance, implying the importance of energy metabolism in cauliflower chromoplast development. Noticeably, Mn superoxide dismutase was a highly abundant protein from cauliflower samples.

In papaya, proteins including methionine synthase, formate dehydrogenase, glutathione-S-transferases, V-ATP synthase, ATP synthase, and adenine nucleotide translocator were detected in high abundance. Methionine synthase is one of the most abundant transcripts in papaya and represents a highly abundant protein induced during ripening (Nogueira *et al.*, 2012). As in the other crop proteomes, ATP synthase and adenine nucleotide translocator were among the most abundant proteins. Glutathione S-transferases (GSTs) belong to a large and diverse group of enzymes in plants. Their localization in plastids has been suggested to play a role in maintaining ROS homeostasis (George *et al.*, 2010). The presence of high abundance of GST implies active redox reaction within papaya chromoplasts.

In pepper, a number of proteins such as ATP-dependent Clp protease, transketolase 1, ketol-acid reductoisomerase, fibrillins, 2-C-methyl-D-erythritol 2,4-cyclodiphosphate synthase (MDS), FtsH-like protein (pftf), capsanthin/capsorubin synthase (CCS), and ATP synthase were present at high

Table 2. Most abundant proteins identified from chromoplast samples of each crop species.

Description	Protein accession	emPAI	Band no.	Amount in gel band (%)	Fraction	Plastid-protein
Watermelon						
Formate dehydrogenase	Cla017090	10.25	6	26.8	S	Yes
Manganese superoxide dismutase	Cla008101	10.11	10	39.1	S	Yes
Cpn20	Cla011138	5.6	10	21.7	S	Yes
V-ATPase subunit A	Cla015485	8.85	16	29.5	M	Yes
Mitochondrial processing peptidase	Cla016811	5.16	18	13.5	M	Yes
ATP synthase beta subunit	Cla003906	6.22	19	28.8*	M	Yes
V-ATPase subunit C	Cla009088	7.67	21	10.5	M	Yes
Adenine nucleotide translocator	Cla022585	10.04	28	30.9*	M	Yes
Putative ADP, ATP carrier	Cla002230	3.37	28	8.9	M	Yes
Annexin	Cla009947	122.87	7, 26	73.0, 55.2	SM	No
Annexin-like protein	Cla009948	82.17	8, 27	56.3, 41.2	SM	No
Universal stress protein	Cla017941	16.71	32	33.8	M	No
HSP15.9	Cla009144	38.62	33	46.5	M	No
Histone H4	Cla005035	12.91	34	17.3	M	No
Tomato						
2-Isopropylmalate synthase	Solyc08g014130.2.1	2.83	12	31.77	S	Yes
V-ATPase subunit B	Solyc01g111760.2.1	4.36	15, 49	19.21, 14.55	SM	Yes
ATP synthase subunit beta chloroplastic	Solyc01g007320.2.1	6.44	15, 50	23.68, 21.74	SM	Yes
Alcohol dehydrogenase-2	Solyc06g059740.2.1	2.94	17	24.05	S	Yes
V-ATPase A1 subunit	Solyc12g055800.1.1	11.55	44	57.51	M	Yes
Mitochondrial processing peptidase	Solyc12g008630.1.1	3.32	48	13.83	M	Yes
ATP synthase beta subunit	Solyc04g007550.2.1	3.72	50	10.36	M	Yes
V-ATPase subunit C	Solyc03g097790.2.1	5.16	54	26.04	M	Yes
ADP, ATP carrier protein	Solyc07g053830.2.1	4	58	5.71	M	Yes
Adenine nucleotide translocator	Solyc11g062130.1.1	3.9	59	9.66	M	Yes
Short-chain alcohol dehydrogenase	Solyc07g047800.2.1	6.09	60	10.32	M	Yes
NADH dehydrogenase	Solyc01g109620.2.1	3.19	61	9.32	M	Yes
Small heat shock protein	Solyc05g014280.2.1	2.82	63	7.5	M	Yes
ADP-ribosylation factor	Solyc01g008000.2.1	5.97	65	13.66	M	Yes
Lipoxygenase homology domain-containing protein 1	Solyc04g054980.2.1	5.96	66	12.33	M	Yes
Lipoxygenase	Solyc01g099190.2.1	2.88	3, 40	56.8, 18.2	SM	No
Lipoxygenase	Solyc08g014000.2.1	3.48	41	35.48	M	No
Late embryogenesis (Lea)-like protein	Solyc01g095150.2.1	4.08	63	12.45	M	No
Stress responsive protein	Solyc01g010750.2.1	5.67	52	16.45	M	No
Histone H4	Solyc04g011390.1.1	86.87	68	60.36	M	No
Cauliflower						
Malate dehydrogenase	Bol039124	17.36	13	49.2*	S	Yes
Mn superoxide dismutase	Bol005872	54.92	46	45.9	S	Yes
V-ATPase subunit A	Bol040479	13.68	20	28*	M	Yes
Mitochondrial processing peptidase	Bol002182	8.31	23	16.8	M	Yes
ATP synthase beta subunit	Bol008794	17.87	24	34.5	M	Yes
ATP synthase subunit gamma	Bol008180	6.5	31	13.6	M	Yes
Adenosine nucleotide translocator	Bol004271	10.9	33	20.5	M	Yes
ATP synthase delta subunit	Bol034235	33.94	35	24.5	M	Yes
60S acidic ribosomal protein P2-2	Bol026417	27.63	41	27.5	M	Yes
Succinyl-CoA ligase beta subunit	Bol038744	7.25	12	16.9	S	No
Histone H4	Bol005544	310.28	45	83.7	M	No
Carrot						
ATP synthase beta subunit	gi 343410685	14.16	10, 40, 41	12.5*, 85.3*, 52.8*	SM	Yes
Cu-Zn superoxide dismutase	gi 76150398	3	27	18.9	S	Yes
V-ATPase catalytic subunit A	gi 137460	7.94	35	34.8	M	Yes
Adenine nucleotide translocator	gi 307135850	4.21	55, 56, 57	40.3*, 30*, 10.4*	M	Yes
NADH dehydrogenase	gi 1171866	2.71	65	11.2	M	Yes
F1-ATPase alpha subunit	gi 34539285	3.46	41	29.2*	M	No

Table 2. (Continued)

Description	Protein accession	emPAI	Band no.	Amount in gel band (%)	Fraction	Plastid-protein
Chain A Calmodulin Isoform 1	gi 178847272	7.94	66	27.3	M	No
Calmodulin	gi 16225	6.21	66	20.6	M	No
Papaya						
Methionine synthase	evm.TU.supercontig_161.10	7.1	5, 36	39.9, 18.7	SM	Yes
Formate dehydrogenase	evm.TU.supercontig_39.83	13.21	18	28.3	S	Yes
Glutathione S-transferase	evm.TU.supercontig_50.117	20.18	23	41.3	S	Yes
Glutathione S-transferase	evm.TU.supercontig_77.114	58.81	24	65.3	S	Yes
Cyclophilin	evm.TU.supercontig_37.181	10.12	29	17.7	S	Yes
V-ATP subunit A	evm.TU.supercontig_155.13	20.58	39	51.8	M	Yes
ATP synthase beta subunit 1	evm.TU.supercontig_65.97	16.05	46	46.6	M	Yes
Adenine nucleotide translocator	evm.TU.supercontig_883.3	8.78	57	17.5	M	Yes
Putative ADP, ATP carrier	evm.TU.supercontig_83.67	3.37	57	6.2	M	Yes
Phospholipase C	evm.TU.supercontig_37.169	13.08	53	26.8	M	No
Annexin	evm.TU.supercontig_4842.1	6.16	55	18.2	M	No
Universal stress protein	evm.TU.supercontig_56.52	24.3	66	43.2	M	No
Pathogenesis-related protein 4	evm.TU.supercontig_1476.3	9.86	68	15.2	M	No
Pepper						
ATP-dependent Clp protease	gi 399213	5.63	6, 40	25.3, 29.2	SM	Yes
Transketolase 1	gi 3559814	7.4	7, 43	32.3, 24.8	SM	Yes
Ketol-acid reductoisomerase	gi 193290660	7.39	11	48.8	S	Yes
4-Methyl-5(b-hydroxyethyl)-thiazol monophosphate biosynthesis enzyme	gi 171854671	5.76	20	27.7	S	Yes
Fibrillin	gi 460761	7.68	23, 52	47.9, 26.7	SM	Yes
2-C-Methyl-erythritol 2, 4-cyclodiphosphate synthase	gi 157042737	11.48	33	34.6	S	Yes
FtsH-like protein	gi 37538489	5.17	44	23.9*	M	Yes
ATP-dependent zinc metalloprotease FTSH	gi 2492515	6.99	44	16.2	M	Yes
zeta-Carotene desaturase	gi 17367814	4.14	46	28.2*	M	Yes
ATP synthase subunit beta	gi 60391817	8.12	48	39.3*	M	Yes
Capsanthin/capsorubin synthase	gi 12643508	43.09	49	95.6	M	Yes
Histone H4	gi 462243	50.9	92	70.2	M	No

* The percentage of homologous proteins identified in the same gel band was grouped as a single family protein. M, membrane fraction of chromoplasts; S, stromal fraction.

abundance. Several of these abundant proteins are also identified in a previous chromoplast proteomics study in pepper (Siddique *et al.*, 2006). CCS catalyses the formation of special pigments and represented the most abundant protein in pepper chromoplast proteome (Table 2). In addition, two other carotenogenic enzymes (i.e. MDS and ζ -carotene desaturase, ZDS) were found to be abundant. Fibrillin represented another most abundant protein. Plastid fusion and/or translocation factor (pfts), a protein involved in plastid membrane biogenesis and originally identified in red pepper (Huguene *et al.*, 1995) was also present at high abundance. Like other chromoplast proteomes, ATP synthase was among the most abundant protein.

Discussion

Chromoplasts from various crops share a similar distribution of functional classes of proteins

This study provides a large-scale proteomic and bioinformatic analyses of chromoplast proteins from six important crop species. Analysis of these six chromoplast proteomic data sets

revealed that the proteins identified had a similar distribution of functional classes, similarly to a previous report (Barsan *et al.*, 2010). A relatively large number of proteins involved in the functional groups including photosynthesis, glycolysis, electron transport/ATP synthesis, lipid metabolism, amino acid metabolism, secondary metabolism, redox, protein metabolism and targeting, signalling, and transport were identified, indicating important roles of these processes in chromoplast development.

The identification of photosynthetic proteins in chromoplasts derived either from chloroplasts or from non-coloured plastids suggests the potential role of maintaining photosynthetic machinery in chromoplasts. All the metabolic precursors used in heterotrophic plastids are either generated by oxidative metabolisms within the organelle or actively imported from the cytosol (Neuhaus and Emes, 2000). Concomitantly, a relatively large number of proteins involved in glycolysis to produce metabolic precursors contributed to the prevalent functional group of chromoplast proteins. In addition, the essential biosynthetic activities in nonphotosynthetic plastids are sustained by the generation of ATP and reducing power (Flugge *et al.*, 2011). A substantial numbers

of proteins involved in energy and reducing power production were observed in these chromoplasts as shown in other studies (Barsan *et al.*, 2010). Chromoplast development is associated with new membrane formation and the formation of lipid-protein carotenoid sequestration structures (Li and Van Eck, 2007). Thus, active lipid metabolism in chromoplasts is expected. Indeed, a recent metabolic study of tomato chromoplasts using radiolabelled precursors revealed that lipid biosynthesis is a very efficient process in chromoplasts (Angaman *et al.*, 2012). Amino acids are synthesized in plastids. The detection of many proteins involved in amino acid biosynthesis from all crops confirmed the process within chromoplasts. Further, chromoplasts contain a highly active redox system. The large number of redox enzyme proteins may allow the plastid to acclimate various processes and signalling during chromoplast development (Egea *et al.*, 2010). While no information is available in documenting protein import machinery in related to chromoplast development, the vast amount of evidence for its role in chloroplast biogenesis (Kessler and Schnell, 2009) suggests the importance of protein import in chromoplast development. Moreover, a relatively large number of proteins involved in signalling and cell metabolism were present in chromoplast proteins, which might play critical roles in the communication between chromoplasts and other organelles. As expected with the nature of heterotrophic plastids, the chromoplast proteomes from all crops contained abundant numbers of transport proteins to provide various substrates and energy supply to chromoplasts.

Early carotenoid biosynthetic and cleavage enzymes are persistently detected in various chromoplast proteomes

As the primary function of chromoplasts is the synthesis and accumulation of carotenoid pigments, carotenoid metabolism is the best-studied metabolic process in chromoplasts. Analysis of various chromoplast samples enabled the identification of many carotenogenic proteins (Fig. 4). A number of early carotenoid biosynthesis proteins prior to lycopene synthesis (i.e. HDS, PSY, PDS, and ZDS) were repeatedly identified from various crop species as shown in other studies (Barsan *et al.*, 2010; Zeng *et al.*, 2011). The detection of these early pathway enzyme proteins may suggest a generally relative abundance of them among the carotenogenic enzymes as well as their central role in directing metabolic flux into carotenoid metabolism. Further, there appears no general correlation between the specific carotenoids accumulated and the presence or absence of up- and downstream enzyme proteins, indicating a complicated regulation of specific carotenoid accumulation in various crops.

Carotenoid-derived metabolites, the apocarotenoids, contribute significantly to flavour and aroma of fruits. Volatile apocarotenoid synthesis increases dramatically during fruit ripening (Simkin *et al.*, 2004; Ibdah *et al.*, 2006). Consistently, CCD1 was detected in nearly all chromoplast samples from these crop samples. Similarly, the key enzyme in the ABA biosynthetic pathway, NCED, was also found from nearly all these species as shown in the *Citrus* chromoplast proteome

(Zeng *et al.*, 2011). The persistent presence of these catabolic enzyme proteins indicates an important role of them in carotenoid metabolism and ripening processes.

Chromoplasts contain high abundance of proteins involved in ATP production and transport

Chromoplasts as heterotrophic plastids require an extensive supply of ATP to energize various metabolic processes in plastids (Neuhaus and Emes, 2000; Egea *et al.*, 2010; Flugge *et al.*, 2011). Among the most abundant proteins identified from each chromoplast sample, ATP synthase and adenine nucleotide translocator were repeatedly observed. Noticeably, the protein gel bands associated with these two proteins represented the most intense bands in the membrane fraction of chromoplasts from all crop species except pepper, in which the strongest bands corresponded to CCS and fibrillin (Fig. 2). High abundance of ATP synthase was also reported in a proteomic study of tomato chromoplasts (Barsan *et al.*, 2012). Although devoid of any photosynthetic activity, chromoplast ATP synthase from daffodil flower is demonstrated to be able to synthesize ATP via the energized chromoplast membrane through the NAD(P)H-dependent respiratory activity (Morstadt *et al.*, 2002). A recent metabolic labelling study also shows an active ATP synthesis in tomato chromoplast (Angaman *et al.*, 2012). Further, a high abundance of ATP synthase is also detected in other heterotrophic plastids, such as in proplastids (Brautigam and Weber, 2009). ATP synthase, which is found among 174 chloroplast phosphoproteins, can be regulated by phosphorylation in controlling its activity (Reiland *et al.*, 2009). Adenine nucleotide translocator facilitates the transport of ATP or ADP across membranes and preferentially imports ATP into heterotrophic plastids, which determines the sink strength of plastids (Neuhaus and Emes, 2000; Flugge *et al.*, 2011). An increased plastidic adenine nucleotide translocator activity has been shown to exert profound effects on lipid and starch synthesis in non-photosynthetic plastids (Tjaden *et al.*, 1998; Reiser *et al.*, 2004).

Membrane proliferation is an important process for chromoplast biogenesis and development (Li and van Eck, 2007). The existence of these two proteins in chromoplast samples could also provide substantial energy for membrane formation in chromoplasts. Active ATP synthesis and the presence of active translocators are known to be needed for massive membrane formation in daffodil flower chromoplasts (Morstadt *et al.*, 2002). The high abundance of these two proteins may suggest the crucial roles of active ATP production and transport during chromoplast development, consistent with the observed elevated energy production components during chloroplast to chromoplast transition in tomato (Barsan *et al.*, 2012).

Highly abundant specific proteins are accumulated in chromoplasts of various crop species

Chromoplasts from various crop species also contain high abundance of other specific proteins. Pepper chromoplasts synthesize the specific carotenoids, capsanthin, and cap-sorubin, which provide the typical colour of red pepper.

Interestingly, while carotenogenic enzymes are generally present at low abundance in plants (Shumskaya *et al.*, 2012), CCS, the enzyme responsible for capsanthin and capsorubin synthesis, represented the most abundant protein in pepper chromoplasts (Fig. 2). Fibrillins are the main structural components of the lipoprotein fibrils and their accumulation directly correlates with carotenoid accumulation in pepper chromoplasts (Deruere *et al.*, 1994). Thus, it was not surprising to find that fibrillins are among the most abundant proteins in red bell pepper chromoplasts. Consistent with a previous study (Siddique *et al.*, 2006), many carotenogenic enzyme proteins were detected abundantly in pepper chromoplasts (Fig. 4). The high abundance of carotenoid biosynthetic pathway enzymes and fibrillins suggests that pepper chromoplasts are characterized with predominant carotenoid biosynthesis and sequestration.

An intrinsic feature of chromoplasts is the presence of a highly active redox system. Watermelon, carrot, and cauliflower accumulated abundant superoxide dismutase. An upregulation of superoxide dismutase activity is observed during chromoplast development (Marti *et al.*, 2009). The activation of plastid superoxide dismutase expression is found to depend on a copper chaperone for superoxide dismutase (Huang *et al.*, 2012). Interestingly, papaya contained a high abundance of GST, which is also found to be a highly abundant protein induced during papaya fruit ripening (Nogueira *et al.*, 2012). GST activity is observed in chloroplasts of *Lycopersicon pennellii* (Mittova *et al.*, 2002). Its plastid localization is suggested to play an important role in maintenance of ROS homeostasis (George *et al.*, 2010). Whether these redox enzymes contribute to the specific characteristics of these chromoplasts remains to be determined.

Some of the detected abundant proteins from various chromoplast samples were predicted to be non-plastidial proteins, which could be due to the tight association of them with chromoplasts or possible dual localization. Indeed, a spinach annexin was found to bind tightly to the outer surface of chloroplasts (Seigneurin-Berny *et al.*, 2000). Approximately 50 plastid proteins have been identified to date as having dual localizations (Carrie *et al.*, 2009). A recent study reveals that mitochondrial processing peptidase is targeted to both mitochondria and chloroplast (Baudisch and Klosgen, 2012). While the presence of these non-plastid proteins could be most likely due to contamination, the high abundance of them in chromoplast samples may exert effects on chromoplast development in specific plant species.

In conclusion, the chromoplast proteomic analysis from six crop species allowed this study to identify large sets of chromoplast proteins with a very good overview of the relatively abundant proteins. These data sets are excellent resources for categorizing chromoplast proteins. This study revealed common metabolic characteristics shared among chromoplasts of different crop species and showed some remarkable differences in relative abundance of some identified proteins among different chromoplasts. Future clarification of the functions and networking of chromoplast proteins will offer new insights into plastid function and contribute to a better understanding of chromoplast biogenesis and development.

Supplementary material

Supplementary data are available at *JXB* online.

Supplementary Fig. S1. Typical HPLC elution profile of carotenoids found in various fruits and vegetables used in this study.

Supplementary Table S1. List of identified proteins and their functional category from each crop species.

Supplementary Table S2. Plastid proteins detected in four or more crops.

Acknowledgements

This work was supported by the USDA-ARS base fund. USDA is an equal opportunity provider and employer. Mention of trade names or commercial products in this publication is solely for the purpose of providing specific information and does not imply recommendation or endorsement by the US Department of Agriculture.

References

- Angaman DM, Petrizzo R, Hernandez-Gras F, Romero-Segura C, Pateraki I, Busquets M, Boronat A. 2012. Precursor uptake assays and metabolic analyses in isolated tomato fruit chromoplasts. *Plant Methods* **8**, 1.
- Balsera M, Soll J, Buchanan BB. 2010. Redox extends its regulatory reach to chloroplast protein import. *Trends in Plant Science* **15**, 515–521.
- Barsan C, Sanchez-Bel P, Rombaldi C, Egea I, Rossignol M, Kuntz M, Zouine M, Latche A, Bouzayen M, Pech JC. 2010. Characteristics of the tomato chromoplast revealed by proteomic analysis. *Journal of Experimental Botany* **61**, 2413–2431.
- Barsan C, Zouine M, Maza E, *et al.* 2012. Proteomic analysis of chloroplast-to-chromoplast transition in tomato reveals metabolic shifts coupled with disrupted thylakoid biogenesis machinery and elevated energy-production components. *Plant Physiology* **160**, 708–725.
- Baudisch B, Klosgen RB. 2012. Dual targeting of a processing peptidase into both endosymbiotic organelles mediated by a transport signal of unusual architecture. *Molecular Plant* **5**, 494–503.
- Berkemeyer M, Scheibe R, Ocheretina O. 1998. A novel, non-redox-regulated NAD-dependent malate dehydrogenase from chloroplasts of *Arabidopsis thaliana* L. *Journal of Biological Chemistry* **273**, 27927–27933.
- Brautigam A, Weber AP. 2009. Proteomic analysis of the proplastid envelope membrane provides novel insights into small molecule and protein transport across proplastid membranes. *Molecular Plant* **2**, 1247–1261.
- Carrie C, Giraud E, Whelan J. 2009. Protein transport in organelles: dual targeting of proteins to mitochondria and chloroplasts. *FEBS Journal* **276**, 1187–1195.
- Chen G, Hackett R, Walker D, Taylor A, Lin Z, Grierson D. 2004. Identification of a specific isoform of tomato lipoxygenase (TomloxC) involved in the generation of fatty acid-derived flavor compounds. *Plant Physiology* **136**, 2641–2651.

- Deruere J, Romer S, d'Harlingue A, Backhaus RA, Kuntz M, Camara B.** 1994. Fibril assembly and carotenoid overaccumulation in chromoplasts: a model for supramolecular lipoprotein structures. *The Plant Cell* **6**, 119–133.
- Eckart K, Eichacker L, Sohr K, Schleiff E, Heins L, Soll J.** 2002. A Toc75-like protein import channel is abundant in chloroplasts. *EMBO Reports* **3**, 557–562.
- Egea I, Barsan C, Bian W, Purgatto E, Latché A, Chervin C, Bouzayen M, Pech JC.** 2010. Chromoplast differentiation: current status and perspectives. *Plant and Cell Physiology* **51**, 1601–1611.
- Flugge UI, Hausler RE, Ludewig F, Gierth M.** 2011. The role of transporters in supplying energy to plant plastids. *Journal of Experimental Botany* **62**, 2381–2392.
- Friso G, Giacomelli L, Ytterberg AJ, Peltier JB, Rudella A, Sun Q, Wijk KJ.** 2004. In-depth analysis of the thylakoid membrane proteome of *Arabidopsis thaliana* chloroplasts: new proteins, new functions, and a plastid proteome database. *The Plant Cell* **16**, 478–499.
- George S, Venkataraman G, Parida A.** 2010. A chloroplast-localized and auxin-induced glutathione S-transferase from phreatophyte *Prosopis juliflora* confer drought tolerance on tobacco. *Journal of Plant Physiology* **167**, 311–318.
- Guo S, Zhang J, Sun H, et al.** 2012. The draft genome of watermelon (*Citrullus lanatus*) and resequencing of 20 diverse accessions. *Nature Genetics* DOI: 10.1038/ng.2470.
- Herman PL, Ramberg H, Baack RD, Markwell J, Osterman JC.** 2002. Formate dehydrogenase in *Arabidopsis thaliana*: overexpression and subcellular localization in leaves. *Plant Science* **163**, 1137–1145.
- Huang CH, Kuo W-Y, Weiss C, Jinn TL.** 2012. Copper chaperone-dependent and -independent activation of three copper-zinc superoxide dismutase homologs localized in different cellular compartments in *Arabidopsis*. *Plant Physiology* **158**, 737–746.
- Hugueney P, Bouvier F, Badiello A, d'Harlingue A, Kuntz M, Camara B.** 1995. Identification of a plastid protein involved in vesicle fusion and/or membrane protein translocation. *Proceedings of the National Academy of Sciences, USA* **92**, 5630–5634.
- Ibdah M, Azulay Y, Portnoy V, et al.** 2006. Functional characterization of *CmCCD1*, a carotenoid cleavage dioxygenase from melon. *Phytochemistry* **67**, 1579–1589.
- Inaba T, Alvarez-Huerta M, Li M, Bauer J, Ewers C, Kessler F, Schnell DJ.** 2005. *Arabidopsis* tic110 is essential for the assembly and function of the protein import machinery of plastids. *The Plant Cell* **17**, 1482–1496.
- Ishihama Y, Oda Y, Tabata T, Sato T, Nagasu T, Rappsilber J, Mann M.** 2005. Exponentially modified protein abundance index (emPAI) for estimation of absolute protein amount in proteomics by the number of sequenced peptides per protein. *Molecular and Cellular Proteomics* **4**, 1265–1272.
- Kessler F, Schnell D.** 2009. Chloroplast biogenesis: diversity and regulation of the protein import apparatus. *Current Opinion in Cell Biology* **21**, 494–500.
- Koumoto Y, Shimada T, Kondo M, Takao T, Shimonishi Y, Hara-Nishimura I, Nishimura M.** 1999. Chloroplast Cpn20 forms a tetrameric structure in *Arabidopsis thaliana*. *The Plant Journal* **17**, 467–477.
- Li L, Van Eck J.** 2007. Metabolic engineering of carotenoid accumulation by creating a metabolic sink. *Transgenic Research* **16**, 581–585.
- Li L, Yang Y, Xu Q, et al.** 2012. The *or* gene enhances carotenoid accumulation and stability during post-harvest storage of potato tubers. *Molecular Plant* **5**, 339–352.
- Lu S, Van Eck J, Zhou X, et al.** 2006. The cauliflower *Or* gene encodes a DnaJ cysteine-rich domain-containing protein that mediates high levels of beta-carotene accumulation. *The Plant Cell* **18**, 3594–3605.
- Marti MC, Camejo D, Olmos E, Sandalio LM, Fernandez-Garcia N, Jimenez A, Sevilla F.** 2009. Characterisation and changes in the antioxidant system of chloroplasts and chromoplasts isolated from green and mature pepper fruits. *Plant Biology* **11**, 613–624.
- Memon AR.** 2004. The role of ADP-ribosylation factor and SAR1 in vesicular trafficking in plants. *Biochimica et Biophysica Acta* **1664**, 9–30.
- Ming R, Hou S, Feng Y, et al.** 2008. The draft genome of the transgenic tropical fruit tree papaya (*Carica papaya* Linnaeus). *Nature* **452**, 991–996.
- Mittova V, Tal M, Volokita M, Guy M.** 2002. Salt stress induces up-regulation of an efficient chloroplast antioxidant system in the salt-tolerant wild tomato species *lycopersicon pennellii* but not in the cultivated species. *Physiologia Plantarum* **115**, 393–400.
- Morstadt L, Graber P, De Pascalis L, Kleinig H, Speth V, Beyer P.** 2002. Chemiosmotic ATP synthesis in photosynthetically inactive chromoplasts from *Narcissus pseudonarcissus* L. Linked to a redox pathway potentially also involved in carotene desaturation. *Planta* **215**, 134–140.
- Neuhaus HE, Emes MJ.** 2000. Nonphotosynthetic metabolism in plastids. *Annual Review of Plant Biology* **51**, 111–140.
- Neuhaus HE, Thom E, Mohlmann T, Steup M, Kampfenkel K.** 1997. Characterization of a novel eukaryotic ATP/ADP translocator located in the plastid envelope of *Arabidopsis thaliana* L. *The Plant Journal* **11**, 73–82.
- Nogueira SB, Labate CA, Gozzo FC, Pilau EJ, Lajolo FM, Oliveira do Nascimento JR.** 2012. Proteomic analysis of papaya fruit ripening using 2DE-DIGE. *Journal of Proteomics* **75**, 1428–1439.
- Plaxton WC.** 1996. The organization and regulation of plant glycolysis. *Annual Review of Plant Biology* **47**, 185–214.
- Reiland S, Messerli G, Baerenfaller K, Gerrits B, Endler A, Grossmann J, Grisse W, Baginsky S.** 2009. Large-scale *Arabidopsis* phosphoproteome profiling reveals novel chloroplast kinase substrates and phosphorylation networks. *Plant Physiology* **150**, 889–903.
- Reiser J, Linka N, Lemke L, Jeblick W, Neuhaus HE.** 2004. Molecular physiological analysis of the two plastidic ATP/ADP transporters from *Arabidopsis*. *Plant Physiology* **136**, 3524–3536.
- Schumacher K, Krebs M.** 2010. The V-ATPase: small cargo, large effects. *Current Opinion in Plant Biology* **13**, 724–730.
- Seigneurin-Berny D, Rolland N, Dorne AJ, Joyard J.** 2000. Sulfolipid is a potential candidate for annexin binding to the outer surface of chloroplast. *Biochemical and Biophysical Research Communications* **272**, 519–524.

Shumskaya M, Bradbury LM, Monaco RR, Wurtzel ET. 2012. Plastid localization of the key carotenoid enzyme phytoene synthase is altered by isozyme, allelic variation, and activity. *The Plant Cell* **24**, 3725–3741.

Siddique MA, Grossmann J, Gruissem W, Baginsky S. 2006. Proteome analysis of bell pepper (*Capsicum annuum* L.) chromoplasts. *Plant and Cell Physiology* **47**, 1663–1673.

Simkin AJ, Schwartz SH, Auldrige M, Taylor MG, Klee HJ. 2004. The tomato carotenoid cleavage dioxygenase 1 genes contribute to the formation of the flavor volatiles beta-ionone, pseudoionone, and geranylacetone. *The Plant Journal* **40**, 882–892.

Tetlow IJ, Bowsher CG, Emes MJ. 2003. Biochemical properties and enzymic capacities of chromoplasts isolated from wild buttercup (*Ranunculus acris* L.). *Plant Science* **165**, 383–394.

The Tomato Genome Consortium. 2012. The tomato genome sequence provides insights into fleshy fruit evolution. *Nature* **485**, 635–641.

Tjaden J, Möhlmann T, Kampfenkel K, Neuhaus Gudrun HaE. 1998. Altered plastidic ATP/ADP-transporter activity influences potato

(*Solanum tuberosum* L.) tuber morphology, yield and composition of tuber starch. *The Plant Journal* **16**, 531–540.

van Wijk KJ, Baginsky S. 2011. Plastid proteomics in higher plants: current state and future goals. *Plant Physiology* **155**, 1578–1588.

Yang Y, Qiang X, Owsiany K, Zhang S, Thannhauser TW, Li L. 2011. Evaluation of different multidimensional LC-MS/MS pipelines for isobaric tags for relative and absolute quantitation (iTRAQ)-based proteomic analysis of potato tubers in response to cold storage. *Journal of Proteome Research* **10**, 4647–4660.

Yang Y, Thannhauser TW, Li L, Zhang S. 2007. Development of an integrated approach for evaluation of 2-D gel image analysis: impact of multiple proteins in single spots on comparative proteomics in conventional 2-D gel/MALDI workflow. *Electrophoresis* **28**, 2080–2094.

Zeng Y, Pan Z, Ding Y, Zhu A, Cao H, Xu Q, Deng X. 2011. A proteomic analysis of the chromoplasts isolated from sweet orange fruits [*Citrus sinensis* (L.) Osbeck]. *Journal of Experimental Botany* **62**, 5297–5309.